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DERMAL INFLUNCE ON EPIDERMAL
RESURFACING DURING THE
REPAIR OF SPLIT THICKNESS
WOUNDS
ANNUAL REPORT

Aug. 15, 1983

DOCUMENT IDENTIFICATION

INVENTORY

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DOCUMENT PROCESSING SHEET

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DERMAL INFLUENCE ON EPIDERMAL RESURFACING DURING
THE REPAIR OF SPLIT THICKNESS WOUNDS

ANNUAL REPORT

Patricia A. Hebda, Ph.D.
William H. Eaglstein, M.D.

August 15, 1983

AD-A175 118

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188
Exp. Date Jun 30, 1986

1a REPORT SECURITY CLASSIFICATION	1b. RESTRICTIVE MARKINGS
Unclassified	

2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	Approved for public release; distribution unlimited

4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION	6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
University of Pittsburgh Office of Research		

6c. ADDRESS (City, State, and ZIP Code)	7b. ADDRESS (City, State, and ZIP Code)
207 Gardner Steel Building Pittsburgh, PA 15260	

8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research and Development Command	8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
		DAMD17-82-C-2149

8c. ADDRESS (City, State, and ZIP Code)	10. SOURCE OF FUNDING NUMBERS			
	PROGRAM ELEMENT NO.	PROJECT NO. 3S162,	TASK NO.	WORK UNIT ACCESSION NO
Fort Detrick, Frederick, MD 21701-5012	62772A	772A874	AA	149

11. TITLE (Include Security Classification)
(U) Dermal Influence on Epidermal Resurfacing During the Repair of Split Thickness Wounds

12. PERSONAL AUTHOR(S)
Patricia A. Hebda, Ph.D. and William H. Eaglstein, M.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 5/15/82 TO 5/15/83	August 15, 1983	

16. SUPPLEMENTARY NOTATION
Part of this work was presented at the 1983 Meeting of the American Society for Investigative Dermatology, Washington, D.C.

17.	COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Dermis; Epidermis; Platelets; Platelet-derived Growth Factor (PDGF); Trauma; Wound Healing
06	05		
06	01		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)
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20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mrs. Judy Pawlus	301-663-7325	SGRD-RMI-S

DD FORM 1473, 84 MAR

83 APR edition may be used until exhausted
All other editions are obsolete

SECURITY CLASSIFICATION OF THIS PAGE

#19 Continued   (Abstract)

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cell outgrowth from explant was measured. Our results are as follows:

1. Whole platelets in phosphate-buffered saline do not increase
    dermal collagen biosynthesis in healing skin wounds.

2. Thrombin-activated platelets cannot be used with the model
    bacause of the apparent systemic effect of the thrombin on
    clotting time.

3. Platelet homogenates (3×10^{10} platelets/ml) produced a slight
    increase in the rate of epidermal resurfacing in healing
    skin.

4. A commercial preparation of platelet-derived growth factor
    (PDGF) stimulated the outgrowth of epidermal cells in explant
    cultures in the presence but not in the absence of fetal
    bovine serum.

5. A platelet homogenate fraction supported epidermal cell
    outgrowth in explant cultures in the absence of fetal bovine
    serum; when added with 5% fetal bovine serum it had a net
    inhibitory effect on outgrowth.

Our conclusions are that:

1. Whole platelet homogenates at high concentration (3×10^{10}
    platelets/ml) show a stimulatory effect on epidermal wound
    healing in vivo, and a complete animal study should be conducted
    to define the effect and assess its significance.

2. A component or components from platelet homogenate supports the
    outgrowth of epidermal cells in skin explant cultures in the
    absence of fetal bovine serum; this component may be different
    from platelet-derived growth factor. In addition, there may
    be another component in platelets that inhibits epidermal cell
    outgrowth at high concentrations. The physiological roles of
    these components may be very important in the normal process
    of wound healing and in the regulation of cellular metabolism
    and will be the subject of further investigation.

ABSTRACT

The purpose of this study is to evaluate dermal-epidermal interrelationships during wound repair by assessing the effect on epidermal wound healing of factors derived from platelets. Two methods were used to approach the problem: 1) a wound healing animal model in which partial thickness excisional wounds made on the backs of young domestic pigs were treated with platelet preparations then evaluated for epidermal resurfacing and dermal collagen biosynthesis; 2) a skin explant culture model in which small sections of partial thickness pig skin (explants) were grown in medium containing fetal bovine serum and/or a platelet fraction, and epidermal cell outgrowth from the explant was measured. Our results are as follows:

1. Whole platelets in phosphate-buffered saline do not increase dermal collagen biosynthesis in healing skin wounds.

2. Thrombin-activated platelets cannot be used with the model because of the apparent systemic effect of the thrombin on clotting time.

3. Platelet homogenates (3×10^{10} platelets/ml) produced a slight increase in the rate of epidermal resurfacing in healing pig skin.

4. A commercial preparation of platelet-derived growth factor (PDGF) stimulated the outgrowth of epidermal cells in explant cultures in the presence but not in the absence of fetal bovine serum.

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Our conclusions are that:

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2. A component or components from platelet homogenate supports the outgrowth of epidermal cells in skin explant cultures in the absence of fetal bovine serum; this component may be different from platelet-derived growth factor. In addition, there may be another component in platelets that inhibits epidermal cell outgrowth at high concentrations. The physiological roles of these components may be very important in the normal process of wound healing and in the regulation of cellular metabolism and will be the subject of further investigation.

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FOREWORD

In conducting the research described in this report, the
investigators adhered to the "Guide for the Care and Use of Laboratory
Animals," prepared by the Committee on Care and Use of Laboratory
Animals of the Institute of Laboratory Animal Resources, National
Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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PROBLEM

This study focuses on epidermal and dermal interrelationships
during wound repair. Platelets contain a growth factor for dermal
fibroblasts. We are testing the effects of platelets on the rate of
epidermal wound healing in an animal model and on the outgrowth of
epidermal cells in a skin explant culture model. The goals of this
study are directed towards obtaining knowledge of the wound healing
process that can be used to develop more effective ways to treat skin
injury.

BACKGROUND

The Skin

The skin is a two-layered covering resting on a subcutaneous
padding of fat. The outer, highly cellular, thin (0.6 to 0.8 mm)
epidermis is in contact with the dermis by way of multiple, irregular
interpapillary ridges (1). These ridges result in increased surface
contact between the two layers; they provide much of the resistance of
normal skin to tangential stress. The innermost portion of the
epidermis is the basal (germinative) layer containing the cells destined
to become the keratinous outer stratum corneum. Cells generated from
the basal layer are gradually extruded towards the surface, forming
first the stratum spinosum (1), characterized by prominent interlocking
cell wall projections which further aid the skin's ability to withstand
shearing forces. The next layer of evaluation is the granular layer
which merges imperceptibly with the clear zone (stratum lucidum). These
layers have a high transferable water content (2), which is intimately
concerned with water retention and heat regulation. These cells
eventually mature and synthesize the outer waterproof fibrous protein
which is keratin. The epidermis, especially the stratum corneum, is the
body's primary defense against penetration by noxious environmental
elements and also prevents the loss of body fluids.

The underlying dermis, which is primarily collagen, elastin, and
proteoglycans, is 20 to 30 times thicker than the epidermis and contains
the nervous, vascular, lymphatic and supporting structures for the
epidermis, as well as harboring the epidermal appendages. Fibroblasts
produce the fibrous proteins, collagen and elastin, which give skin its
strength (3). Mast cells containing histamine and heparin produce the
proteoglycans (ground substance) (4) which form the interfibrillary
matrix of the dermis. Tissue macrophages are distributed around blood
vessels and hair follicles (5). The appendages of human skin include
hair follicles and their associated sebaceous glands, the eccrine sweat
glands which enter through interpapillary ridges, and the aprocrine
glands located in the axillary and inguinal regions (6).

The Wound Healing Process

When a transverse disruption of the skin occurs, one of the first
problems for the host is to re-establish an epidermal continuum. In
mammals, this is accomplished by the mobilizaiton of epidermal cells at

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the edges of the defect and their migration across the viable tissue in the wound bed (7). When epidermal appendages are involved, they also serve as a source of mobilizable cells. As the epithelial continuum is re-established, cell loss is replaced through a burst in DNA synthesis and mitosis. Peak mitotic activity occurs at approximately 28 to 72 hours after wounding irrespective of the status of the epithelial closure (8). Increased mitotic activity extends beyond the actual defect for as much as 6 mm into the uninvolved epidermis (9). Just as the migrating activity proceeds until closure, the mitotic response will reach higher peak activities and will persist above baseline value for a longer period of time in wounds presenting more tissue destruction. It is thought that some type of negative feedback mechanism controls this response. Once the defect is covered, there is a process of differentiation, whereby the epithelial cells synthesize keratin and ultimately re-establish the various structural relationships and strata within the epidermis.

Dermal repair occurs primarily by connective tissue proliferation, rather than by regeneration of the original anatomical structures. Initially, there is a polymorphonuclear neutrophylic leukocyte infiltration into the wounded dermis (10). Cells of the monocyte-macrophage series are then seen to dominate at a period of 48 to 72 hours (11). These so-called wound macrophages are chemotactic to fibroblasts and are instrumental in their mobilization to the site of injury (12). After macrophages arrive, the number of fibroblasts begins to increase. The fibroblasts lay down the first significant amount of collagen, the fibrous protein that is responsible for much of the structural integrity of the tissue. The first collagen which is synthesized is embryonic-type collagen (Type III) (13). It consists of three identical α_1 chains held together in a helical conformation by hydrogen bonds and disulfide cross-links. As the wound matures, Type III collagen is gradually replaced by adult-type collagen (Type I, $2\alpha_1$, $1\alpha_2$). As Type I collagen is made, there is a substantial increase in strength of the wounded dermis. The gain in mechanical strength is due to extensive remodeling of the collagen molecules (14). This remodeling involves closer packing of the collagen fibrils and organization into large bundles due to extensive intermolecular cross-linking.

Generally, wound healing has been investigated as either a dermal or epidermal process depending upon the interest of the investigator. A multiplicity of quantitative methods for measuring the repair of a dermal wound can be found in the literature, but nearly all of these methods rely on parameters of dermal healing, e.g., gain in tensile strength (15-18), hydroxyproline content to reflect collagen synthesis (19-23), hexosamine content (24), weight of granulation tissue, DNA content to reflect fibroplasia and others (25). The parameters for measuring the events in epidermal wound healing are fewer and not as well defined. The dynamics of epidermal healing can be divided into three phases: epithelial migration to cover the defect; cell division to supply new cells replacing those lost by wounding; and differentiation of a new epidermis. Numerous investigators have described the epithelialization of cutaneous wounds by histological examination (26-28). Only recently, however, have some of the

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ultrastructural aspects of repairing the skin been reported (29-31). By the use of tritiated thymidine or colchicine derivatives, some information about the cell kinetics in human wound healing has been reported (32-35). The morphological return of a differentiated (mature) epidermis following wounding has been described and can be correlated mostly with the return of a physiological parameter, namely water barrier function which can be measured by electrical hygrometry (2,36,37).

These investigations have yielded important information concerning a solitary phase of the healing process. However, wound healing is a sequence of events whereby epidermal and dermal repair occur simultaneously.

Dermal-epidermal Interactions

The influence of dermal collagen on epidermal cell function has received a great deal of experimental and speculative attention. By grafting recombinants of separated epidermis and dermis from different regions of adult guinea pig back to the animal for prolonged periods, Billingham and Silvers (38) showed that the dermal component determined the specific regional characteristics of the overlying epidermis. Briggaman and Wheeler (39) studied the role of dermis on epidermal maintenance by grafting recombined epidermis and dermis to the chorioallantoic membrane of an embryonated chicken egg and found that epidermal maintenance was dependent on the presence of the dermis. They showed that: 1) both viable and freeze-thawed dermis as well as homologous and herterologous dermis maintained epidermis in recombinants and 2) other substrate materials (millipore filter, agar gel, and heat killed dermis) were not effective in maintaining epidermis. We know that in vitro epidermal cells grow better on collagen coated culture dishes than on noncoated surfaces (40) and that they preferentially attach to molecularly distinct collagen Type IV (41,42). Recently Baden et al. (43) detected a modification of polypeptide composition in keratinocyte fibrous protein (keratin) due to changes in the dermis beneath the keratinocyte. These investigators observed different polypetide patterns in scarred skin, thus indicating that the nature of the dermal connective tissue can influence epidermal cell keratin synthesis. Our basic research investigations have shown that if a proline analogue is topically applied to the wound, there is an inhibition of dermal collagen biosynthesis and subsequently a delay in re-epithelialization. The data suggest that dermis is important for not only the maintenance but also for at least some of the behavior of epidermis. The manner in which the dermis exerts this influence has not been elucidated and remains an intriguing problem.

During the past few years, we have been investigating the mechanisms involved in epidermal wound healing (migration, mitosis, maturation). In 1978 we designed an animal wound healing model for the evaluation of epidermal repair in split thickness skin wounds (44). We use young domestic pigs because pig skin resembles human skin in the following parameters: relative thickness of the epidermis and dermis, relative sparsity of hair and the presence of a deep layer of subdermal fat (7). Using this wound healing model, we have been successful in

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making certain observations on the healing of superficial wounds that have directly led to improvements in their treatment (45). We have expanded our original wound healing model so that it is possible to evaluate both re-epithelialization and dermal repair in one wound at one time. We have used this expanded model to study several aspects of dermal and epidermal interrelationships during the wound healing process (45a).

Platelets and Platelet-derived Growth Factor

Ross and co-workers (46) were the first to demonstrate that platelets stimulated the proliferation of smooth muscle cells in vitro. These investigators found that dialyzed serum from clotted monkey blood promoted the proliferation of monkey arterial smooth muscle cells in culture, but dialyzed serum prepared from recalcified platelet-poor plasma was much less effective. The addition of platelets and calcium to platelet-poor plasma increased the activity of the plasma to the same level achieved with blood serum. Furthermore, the addition to plasma of a platelet-free supernatant solution prepared by exposing purified platelets to thrombin also stimulated the proliferation of smooth muscle cells. They concluded that much of the growth-promoting activity of dialyzed serum was directly or indirectly derived from platelets. Subsequently, Harker et al. (47) in in vitro experiments demonstrated that purified platelets were mitogens that stimulated the proliferation of smooth muscle cells and that the stimulatory effect could be prevented by drug-induced platelet dysfunction. Balk et al.(48) noticed the same effect in chicken fibroblasts. Burke and Ross (49) found that the proliferative effect of purified platelets was not specific to smooth muscle cells and that the stimulatory effect was characterized by a dramatic increase in collagen biosynthesis. Eichner, Cardinale and Fuller (50) examined the effect of platelet extract on collagen synthesis using rabbit aortic smooth muscle cells in culture and found that the platelets stimulated collagen synthesis specifically. Recently Buckingham (personal communication) observed significant stimulation in collagen biosynthesis when fibroblasts from human skin were incubated with purified platelets; he and his collaborators found that the addition of platelets to fibroblasts incubated in already platelet-rich blood serum resulted in increased collagen biosynthesis. More recently, Bankowski and Dabrowski (51) demonstrated that an addition of platelet homogenate to confluent cultures of 3T3, L-929, and PZ-32 cells increased significantly the incorporation of proline into proteins and the synthesis of the hydroxyproline-containing, collagenase-sensitive proteins. These elegant experiments demonstrated that platelets or their components directly stimulate fibroblast collagen production.

Thus far, experimental studies have focused on the cationic factor from platelets first described by Westermark and Wasteson (52,53) called the platelet-derived growth factor (PDGF). This factor has been purified and extensively characterized by Antoniades (54). PDGF has been shown to stimulate cells of mesodermal origin and a cell surface receptor has been identified in responsive cells (55). Several other growth-promoting factors have also been found in platelets. These include PBP (platelet basic protein) (56) which is a mitogen for Swiss 3T3 cells and CTAP III (connective tissue activating peptide III) shown

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by Castor et al (57) to stimulate human synovial cells and dermal fibroblasts (for a recent review see Ref. 58).

Because platelets are in the blood and respond to injury with a specific release reaction, it has been speculated that platelets contain a "wound hormone". Such a hormone would signal cells to begin to synthesize repair connective tissue. The platelet-derived growth factor (PDGF) is an appealing candidate for the role of wound hormone since it stimulates smooth muscle cells and fibroblasts to proliferate and to synthesize collagen.

To our knowledge, no one has ever used platelet extract to stimulate wound healing in vivo. However, Knighton et al (59) report a single clinical case of a patient with platelet dysfunction (thrombocytopenia secondary to myelofibrosis) who developed a non-healing wound after a biopsy. After trying several supportive therapies with no effect, they gave a single platelet transfusion and soon observed granulation tissue in the wound bed. The wound then progressed through the normal healing process over a period of three weeks. This clinical finding supports the theory that platelets play an essential role in the healing process.

APPROACH

The following experiments were designed to examine the effects on the epidermis of adding a factor that affects the dermis (factors from platelets). Two methods were used to test for a stimulation of epidermal wound healing by platelets—an in vivo wound healing study using an animal model (44) and an in vitro skin explant culture study (60).

Animal Wound Healing Model

Because we want to study both the dermis and the epidermis simultaneously during wound healing, we have chosen to study partial thickness wounds. A partial thickness wound is characterized by disruption of epidermal continuity, loss of all the surface epidermis and destruction of the papillary and superficial reticular dermis. Much of the follicular, glandular and ductile epithelial cells within the dermis are not destroyed. These epithelial cells and ones from the wound edge migrate and resurface the wound (Figs. 1-4).

Young, white Yorkshire pigs 20-30 lbs and approximately two to three months old were used for this study. The pigs were housed individually in our animal facility with controlled temperature (19-21°C) and light (12/12 LD) and were fed a basal diet ad libitum. Although complete environmental studies on wound healing in pigs have not been done, these conditions have yielded reproducible results in our model.

The pigs were clipped with standard animal clippers and any remaining hairs were gently removed with a razor. The skin on the entire back and sides was prepared for wounding by washing with mild
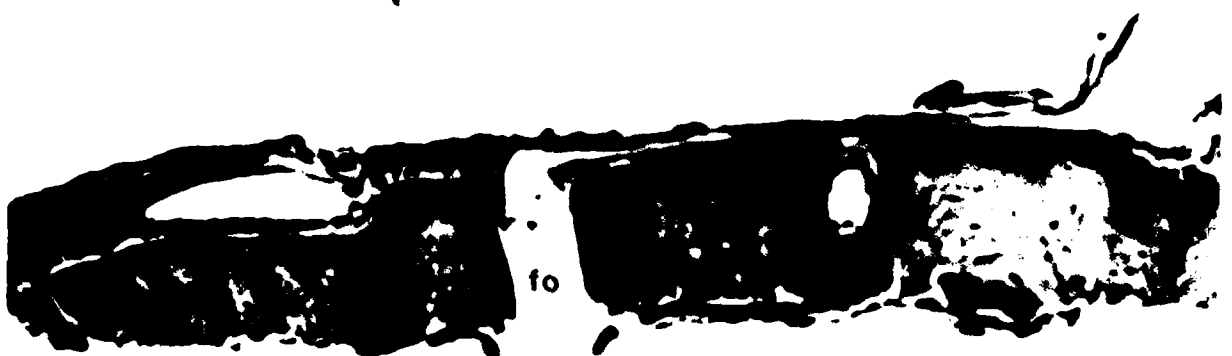
FIGURE 1: Photomicrograph (x 46) of the portion of skin removed when wounding. Note that the entire surface epidermis (e) and papillary dermis (d) including the follicular orifices (fo) are removed.

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FIGURE 2:   Photomicrograph (x 46) of the wound bed immediately after

wounding.   Note the islands of epithelial cells within the

dermis (†).   Re-surfacing occurs mostly from the follicular,

glandular and ductile epidermal reserviors within the dermis

and partly from the wound edge.

FIGURE 4:   Photomicrograph (x 46) of resurfaced epidermis. Note the

migrating epithelium (e) is invading the underlying connective

tissue demarcating necrotic material which forms the crust (c).

soap and water and rinsing with water and 70% alcohol. Other antiseptics were not used because of the potential effect on the healing process. For this series of experiments the pigs were 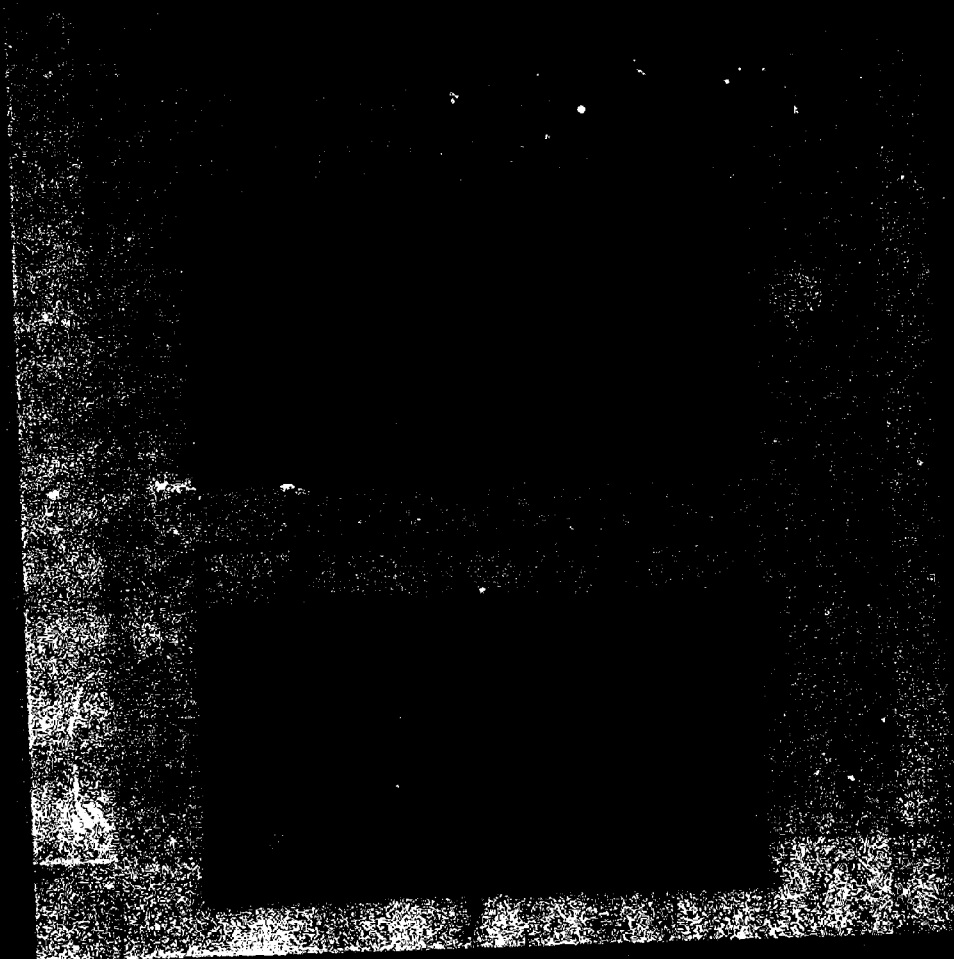anesthetized (sodium Nembutal, i.p. and/or i.v.) and approximately 85 rectanglar 7 x 10 mm wounds, 0.3 mm deep were made in the paravertebral and thoracic areas with a Castroviejo dermatome (Fig. 5). The wounds on each animal were divided into five treatment groups and treated with various concentrations of an aqueous platelet preparation as indicated in Table I. The aqueous platelet preparation was allowed to rest undisturbed on each wound for five minutes, then excess treatment was gently blotted off. Some wounds were sampled by excision daily for the next five days. Using the Castroviejo dermatome skin samples 22 x 33 mm, 0.5 mm deep, that included the entire wound and some surrounding normal tissue were removed. These specimens were incubated in 0.25% trypsin at 4°C for 18 h to separate the dermis and epidermis. The epidermal specimens were mounted on cardboard for evaluation and the dermal specimens were placed in RMPI 1640 medium containing 5% fetal bovine serum (FBS) and stored at -10°C until they were assayed for collagen biosynthetic capacity.

The aqueous platelet preparation was prepared three different ways for testing: 1) fresh washed platelets were suspended in phosphate-buffered saline and used for treatment; however, our in vitro studies indicated (see below) as did the work of others (59) that whole platelets do not stimulate fibroblasts, but need to be activated. Therefore, we modified our procedure to use; 2) thrombin-activated platelets; and 3) platelet homogenates. In both of these activated preparations the platelet-derived growth factor is in a soluble form more readily accessible to the wounded tissue.

Epidermal specimens were evaluated for healing by the method devised by Eaglstein and Mertz (44) (see background). After the dermis and epidermis were incubated with trypsin, the two layers were easily separated with forceps. The moist epidermis was placed on a microscope slide with the stratum corneum (and protruding hair shafts) against the glass. The slide and specimen were pressed against cardboard and the slide carefully removed to leave the epidermis on the cardboard. The specimen was carefully examined for any defect (hole) in the area of the wound. If a crust was present (Days 3-5) this was gently removed with forceps to observe the underlying newly formed epidermis. If a hole is present, the wound is designated not healed. If no defect is found, the wound is healed (See Fig. 6). An HT_{50} value (time at which 50% of the wounds are healed) is calculated for each experimental group and these values obtained with platelet preparations are compared with vehicle treated and untreated control values to determine whether epidermal healing has been enhanced.

Dermal specimens were evaluated for their capacity to synthesize collagen using a method described by Diegelmann et al., (22) adapted for analysis of healing wounds by Alvarez (unpublished). However, during the course of this study, it became evident that a problem existed with the assay technique and the entire procedure was re-evaluated (see Results and Discussion). In its final form the assay was performed as follows: The dermal wound bed and 1-2 mm of surrounding dermal tissue

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FIGURE 5: Photograph of Experimental Animal Immediately After Wounding.
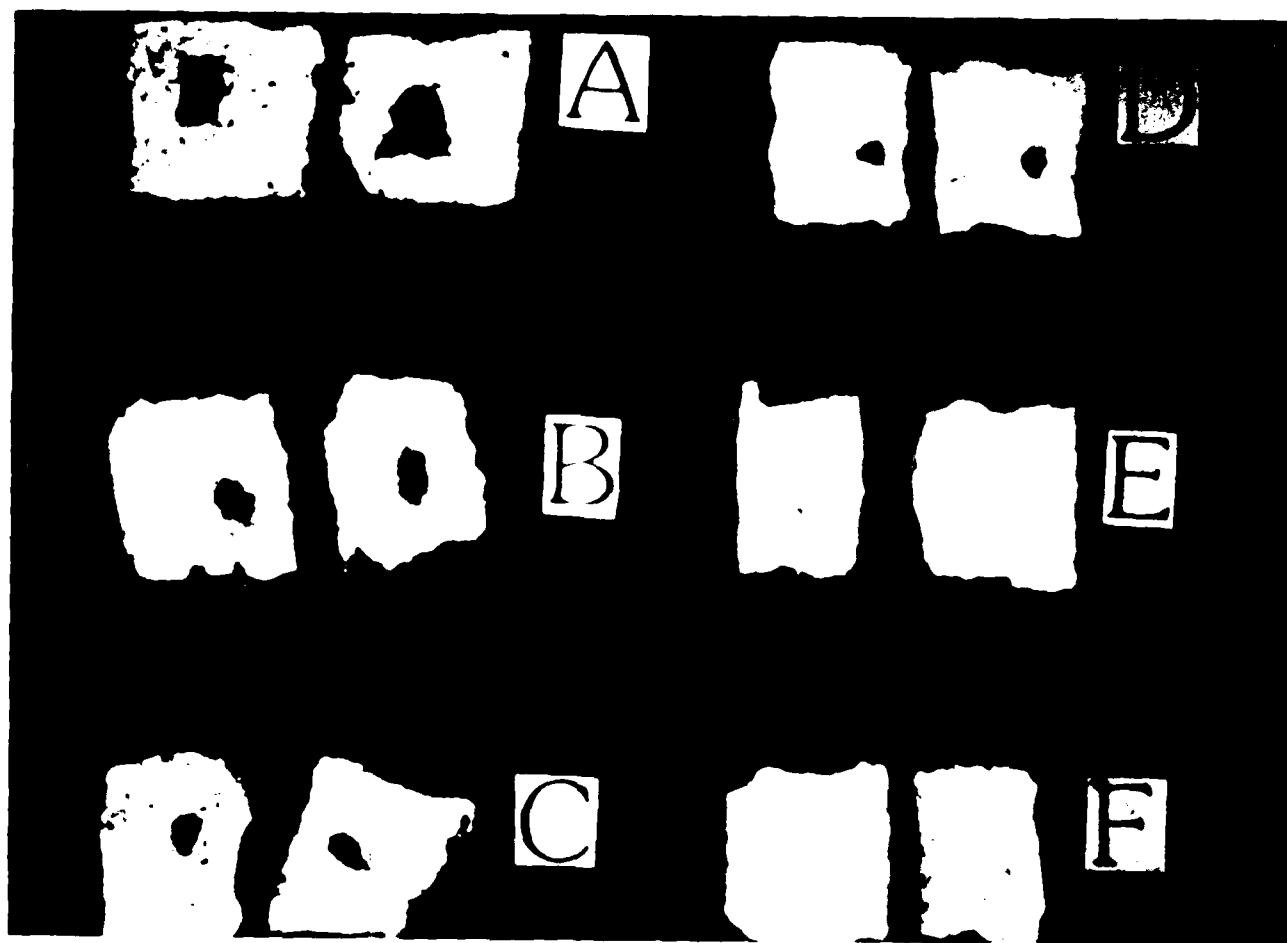
FIGURE 6: Separated Epidermal Specimens Containing Wound Sites

A - Day 2 Not Healed
B - Day 3 Not Healed
C - Day 4 Not Healed
D - Day 5 Not Healed
E - Day 6 Healed
F - Day 7 Healed

were cut from the excised dermal specimen with a sharp scalpel. Two to three specimens were combined for each analysis. The tissue was minced on a sterile plastic surface using two sharp scalpels in a scissor-like motion. The minced tissue was incubated in 4 ml Krebs-Ringer solution without phosphate (20) containing [^{14}C]-proline (2 µCi/sample) at 37°C for 18 h After incubation, protease inhibtors (N-ethylmaleimide, phenylmethyl sulfonylfluoride, and ethylene diamine tetracetic acid) were added and the reaction was stopped by freezing. Radiolabelled collagen was assayed according to Hebda et al (Appendix 1). Each sample was homogenized and dialyzed to remove unincorporated label. Then it was hydrolyzed in 6N HCl under atmospheric N$_2$ for 24 h and lyophilized. The hydrolysate was neutralized, dansylated and chromatographed on a thin-layer chromatography sheet in a solvent system which separates dansyl-proline, dansyl-hydroxyproline and the free imino acids. Radioactive areas were cut out and counted in a liquid scintillation counter and the relative collagen biosynthesis (RCB) was calculated using Peterkofsky's equation (61).

Skin Explant Culture Model

Our platelet preparations (as described above) and a commercial preparation of PDGF (Bethesda Research Labs, Bethesda, MD) were tested for their effects on epidermal growth in a skin explant system. The method of preparing explants was first described by Halprin (60). Essentially, the method serves as an in vitro assay for epidermal cell migration and mitosis and has been used to identify agents that influence epidermal wound healing (directly or via the underlying dermis). With a Castro-viejo dermatome strips of skin (22 x 100 mm, 0.2 mm thick) were excised steriley from the back of a pig. These were cut using a very sharp scalpel into pieces 1-2 mm on a side which were placed dermal side down in 35 cm^2 plastic culture dishes (4 explants/dish), allowed to air dry for about 20 min, then incubated in 2 ml RPMI 1640 medium containing 2% antibiotic/antimycotic solution and fetal bovine serum (FBS) and/or a platelet-derived fraction. Outgrowth was evaluated by measuring the largest radius of the epidermal sheet. In this system fibroblasts may also begin to grow from the dermal layer but they did not present a problem because: 1) they are readily distinguishable from epidermal cells under the microscope—epidermal cells grow as a continuous coherent sheet, whereas fibroblasts are seen as individual cells and 2) fibroblasts do not begin to grow until 6 or 7 days of culture and epidermal cells from our explants began to grow usually within 24 h, the critical time of evaluation was found to be the first 5 to 7 days (see Results and Discussion).

Outgrowth of epidermal cells was measured under a tissue culture microscope fitted with a micrometer eyepiece. The living explant culture was examined under the microscope for the maximum radius of epidermal sheet. The length of time the culture dish was out of the incubator was 30 min or less.

RESULTS AND DISCUSSION

Wound Healing Animal Model

These preliminary studies were conducted to determine the most effective concentration of platelets and frequency of applications. Platelet preperations described below were applied topically to wounds according to a regimen outlined in Table I.

A. Platelet Preparations.

1. Fresh platelet suspensions. Recently outdated human platelets (> 1 week old) were obtained from the blood bank and collected by centrifugation (100 x g for 15 min). When necessary, red blood cells were first removed by low speed centrifugation (400 x g for 10 min). The platelets were washed once with phosphate-buffered saline and suspended in phosphate-buffered saline for application. Two separate experiments were conducted using a total of four animals. Two or three wounds per group were sampled each day for 5 days. Collagen biosynthesis was measured in trypsin-split dermal samples. No difference was found in percent RCB (relative collagen biosynthesis) between platelet-treated, vehicle treated on untreated groups. We interpret this to indicate that platelets were not releasing their growth factors at the site of injury.

2. Thrombin-activated platelets. Thrombin (10 units/ml) was added to fresh platlet suspensions, causing the platelets to aggregate (and release the contents of their α-granules). The suspensions were then applied to wounds as before. Two or three wounds per group per day were sampled by excision. In both experimental animals abnormal bleeding after excision on days 2-5 was observed; i.e. clotting time was delayed. We attribute this to a systemic depletion of 'serum clotting factors by the thrombin, since we have never observed such a response before or since. Therefore, thrombin was found to be an unacceptable activator of platelets.

3. Platelet homogenate. Homogenization has been used to solubilize PDGF (51). We homogenized fresh platelets with a tissuemizer and applied the homogenates to wounds as previously described. Samples were taken for analysis of epidermal healing and dermal collagen biosynthesis (five wounds per group per day). The dermal samples were lost due to a power failure which interupted refrigeration. Epidermal samples were evaluated and we found a slight decrease in healing time for group I (3 x 10^{10} pl/ml) compared with group IV (vehicle). The results, shown in Table II, will be used to design a complete evaluation of wound healing using four animals and sampling wounds daily for seven days.

TABLE I: TREATMENT OF WOUNDS DURING DOSE/FREQENCY RESPONSE STUDIES

Group No.	Treatment[a]
1	3×10^{10} pl/ml[b] in phospate-buffered saline
2	3×10^9 pl/ml in phosphate-buffered saline
3	3×10^8 pl/ml in phosphate-buffered saline
4	vehicle - phosphate-buffered saline
5	control - untreated

[a] In each series pig 1 was treated daily, pig 2 was treated only on day of wounding.

[b] Platelets/ml; the platelets were activated in the second series with thrombin (10 units/ml), in the third series by homogenization.

TABLE II: Rate of Epidermal Wound Healing with Platelet Homogenates.

Group	Treatment	HT_{50}[a] (Days)
I	3×10^{10} pl/ml	4.7
II	3×10^{9} pl/ml	> 5
III	3×10^{8} pl/ml	> 5
IV	vehicle	> 5
V	untreated	> 5

[a] Time at which 50% of the wounds are healed.

B. Collagen Biosynthesis Assay:

When dermal specimens were assayed according to the method of Diegelmann (22) the following problems were identified and resolved:

1. We found that incubation of dermal samples with radioactive proline at 37°C for 3 h was insufficient for obtaining enough labelled protein. By conducting a time course study of incorporation of radioactive proline we found that 10-24 h was required for adequate labelling. We now incubate samples at 37°C for 18 h (overnight). We found no difference in percent labelling whether the samples were incubated with or without shaking.

2. During TCA precipitation steps, we had difficulty in removing the supernatant solution without inlcuding small amounts of precipitate. We therefore, used dialysis vs tap and distilled water to remove unincorporated label.

3. In the initial assay collagen was measured by its sensitivity to purified collagenase. Labelled protein is incubated with collagenase and the low molecular weight products are separated by dialysis. In our system this technique presented some difficulties. Different results were obtained with different commercial collagenases. The most selective enzyme preparation (i.e. the one yielding the lowest percent labelled material in the dialysate is "Collagenase Type III" prepared by Advance Biofactures Corporation (New York). This is the enzyme we now use when we assay for collagenase sensitive protein. However, we are routinely using a more facile assay system which measures hydroxyproline as an estimation of collagen (see Hebda et al Appendix 1). Results obtained are comparable to those obtained using highly purified collagenase and we find the hydroxy-proline assay simpler and more economical for multiple samples.

Skin Explant Culture Model

Skin explants (0.2 mm thick) from young Yorkshire pigs were grown for 5 days in RMPI 1640 supplemented with varying concentrations of FBS (0-20%) and a commercial preparation of PDGF (0-5 units/ml). The results, shown in Tables III and IV, indicate that PDGF enhances epidermal outgrowth in low and high concentrations of FBS, but not in the absence of serum. Maximum outgrowth occurred with 15% FBS and 1.0 to 2.0 units/ml commercial PDGF; outgrowth was about 42% greater than with 15% FBS alone (p < 0.01). These findings suggest that PDGF may be important (directly or indirectly) in epidermal cell outgrowth.

During this series of experiments we encountered several difficulties which we have attempted to correct. The first was that the percentage of viable growing explants was about 25% of the total cultured. Since some antiseptic agents can leave a residue on the skin which may inhibit cell growth we stopped using these to sterilize the pig skin before excision; instead we scrubbed well with mild neutral pH

TABLE III: 5-DAY OUTGROWTH OF EXPLANT CULTURES WITH FETAL BOVINE SERUM

Conc. of FBS	EXPERIMENT NO. 1			EXPERIMENT NO. 2		
	Number of Samples	Outgrowth (Mean ± S.E.) µm	Relative Outgrowth (% ± S.E.)	Number of Samples	Outgrowth (Mean ± S.E.) µm	Relative Outgrowth (% ± S.E.)
0%	8	544 ± 31	67 ± 3.8	--	--	--
5%	9	810 ± 65	100 ± 8.1	47	877 ± 17	100 ± 1.9
10%	30	944 ± 175	116 ± 21.6	25	932 ± 33	106 ± 3.8
15%	46	821 ± 39	101 ± 4.8	4	566 ± 65	64 ± 7.5
20%	--	--	--	4	855 ± 102	97 ± 11.6

TABLE IV: 5-DAY OUTGROWTH OF EXPLANT CULTURES WITH PLATELET-DERIVED GROWTH FACTOR

Conc of PDGF units/ml*	EXPERIMENT 1			EXPERIMENT 2		
	Number of Samples	Outgrowth (Mean ± S.E.) μm	Relative Outgrowth (% ± S.E.)	Number of Samples	Outgrowth (Mean ± S.E.) μm	Relative Outgrowth (% ± S.E.)
(+0% FBS)						
0.2	67	No Outgrowth				
2.0	71	No Outgrowth				
5.0	61	No Outgrowth				
(+5% FBS)						
0	9	810 ± 65	100 ± 8.1	47	877 ± 17	100 ± 1.9
0.2	19	932 ± 53	115 ± 6.6	31	777 ± 30	89 ± 3.4
1.0	--	--	--	26	910 ± 42	104 ± 4.7
2.0	28	977 ± 26	120 ± 3.2	3	733 ± 125	84 ± 14.3
5.0	63	999 ± 21	123 ± 2.6	7	733 ± 44	84 ± 5.1
(+15% FBS)						
0	15	955 ± 71	100 ± 7.4	4	566 ± 65	100 ± 11.6
0.2	14	777 ± 57	81 ± 5.9	1	710	125
1.0	54	1365 ± 48	143 ± 5.0	10	744 ± 28	131 ± 4.9
2.0	--	--	--	4	832 ± 28	147 ± 4.9
5.0	31	977 ± 52	102 ± 5.5	5	832 ± 63	147 ± 11.1

* 1 unit/ml PDGF = The amount which evokes a response in fibroblasts equal to that of 5% FBS.

soap and water and rinsed extensively with 70% alcohol and sterile water. The second was that the commercial preparation of PDGF was not consistently active, as determined by running a standard assay for mitogenic effect on L-929 fibroblasts (see Appendix 2). Comparing the results in Table III between experiments 1 and 2, it seems probable that the differences in relative outgrowth may be due to a lower activity of PDGF in experiment 2. Since the commercial PDGF's potency was inconsistent we decided to conduct studies with a freshly prepared fraction from washed platelets. Efforts were made to correct these difficulties. This modification improved our percentage of viable growing explants to over 90% in control cultures. The platelets were suspended in phosphate-buffered saline, homogenized and centrifuged (10,000 x g for 10 min). The supernatant solution was sterilized by passage through a 0.4 m filter then added to RPMI 1640 culture medium. This platelet homogenate fraction (PHF) was mitogenic for fibroblasts.

Two experiments were conducted incorporating the above modifications (omitting antiseptics other than alcohol and using PHF). Explants were grown with FBS (0-15%) and PHF (0-262 g protein/ml). Cultures were evaluated for outgrowth after three days and those explants that had epidermal cell outgrowth on three or four sides were designated as growers. Their outgrowth was measured on days 5 and 7. The results, shown in Table V, demonstrate that the PHF is capable of substituting for FBS in stimulating and supporting outgrowth of epidermal cells. Some outgrowth in the absence of FBS and PHF was observed on Day 3 but only in 2 out of 124 explants and the outgrowth stopped after 5 days. This observed outgrowth may be due to residual nutrients present in the explant to migration (requiring less energy) rather than mitosis since it occurs early in culture.

Figure 7 shows the results of outgrowth when FBS and PHF were added alone and in combination. There is no significant difference in outgrowth between 5, 10, and 15% FBS. Nor is there a difference in outgrowth between the three concentrations of PHF. We interpret this lack of difference to mean that the stimulatory factor is not the rate-limiting component in 5% FBS or 26 g/ml PHF. However the radius of outgrowth in the presence of PHF is slightly less than the radius of outgrowth with FBS (p=0.01). The rate of outgrowth (slope) is the same for these two groups between days 3 and 7. The results of adding PHF and FBS together are very dramatic. The total outgrowth and rate of outgrowth both are less than with either one alone. This finding strongly suggests the presence of an inhibitor of epidermal cell outgrowth in blood platelets. When the concentration of this component is high, as when PHF and PBS are combined in the culture medium, it counteracts the effect of the stimultory factor. This inhibitor may be acting as a toxin for epidermal cells or it may have a more specific effect such as blocking the influence of the stimulator. The second case would be more interesting in that it suggests a regulatory function for the inhibitor. Such an agent could serve to control epidermal cell proliferation.

Since serum is derived from whole clotted blood it contains factors released from platelets. The results of these experiments demonstrate that a factor or factors are present in platelets which support the

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TABLE V: OUTGROWTH FROM EXPLANTS WITH FETAL BOVINE SERUM AND PLATELET HOMOGENATE FRACTION

Treatment	Experiment 1				Experiment 2			
		Outgrowth ± S.E. (μm)				Outgrowth ± S.E. (μm)		
	n/T^a	Day 3	Day 5	Day 7	n/T^a	Day 3	Day 5	Day 7
FBS^b								
0%	0/44	--			2/80	366 ± 79	516 ± 17	522 ± 11
5%	36/40	684 ± 19	768 ± 21	882 ± 18	44/80	568 ± 23	755 ± 24	826 ± 22
10%	44/48	652 ± 14	739 ± 14	841 ± 20	9/64	534 ± 50	781 ± 46	963 ± 31
15%	33/48	645 ± 21	781 ± 23	898 ± 31	22/68	545 ± 31	733 ± 20	895 ± 27
PHF^c (μg/ml)								
26	33/40	608 ± 18	747 ± 21	846 ± 26	44/72	534 ± 28	713 ± 27	772 ± 24
131	24/40	555 ± 22	689 ± 20	808 ± 24	30/80	495 ± 29	695 ± 36	827 ± 39
262	33/40	500 ± 21	604 ± 16	673 ± 28	31/76	386 ± 31	664 ± 21	834 ± 39
FBS + PHF								
5% 26	7/36	254 ± 27	326 ± 29	374 ± 28	28/80	216 ± 9	311 ± 14	394 ± 18
5% 131	17/48	190 ± 14	249 ± 17	321 ± 17	15/76	233 ± 11	284 ± 20	361 ± 32
5% 262	21/40	229 ± 19	279 ± 17	385 ± 29	23/80	253 ± 16	300 ± 14	381 ± 19

a Number of samples evaluated/Total number of explants prepared

b Fetal Bovine Serum

c Platelet Homogenate Fraction (See Text)

FIGURE 7: EPIDERMAL OUTGROWTH IN EXPLANT CULTURES GROWN WITH FETAL BOVINE SERUM (FBS) AND PLATELET HOMOGENATE FRACTION (PHF)
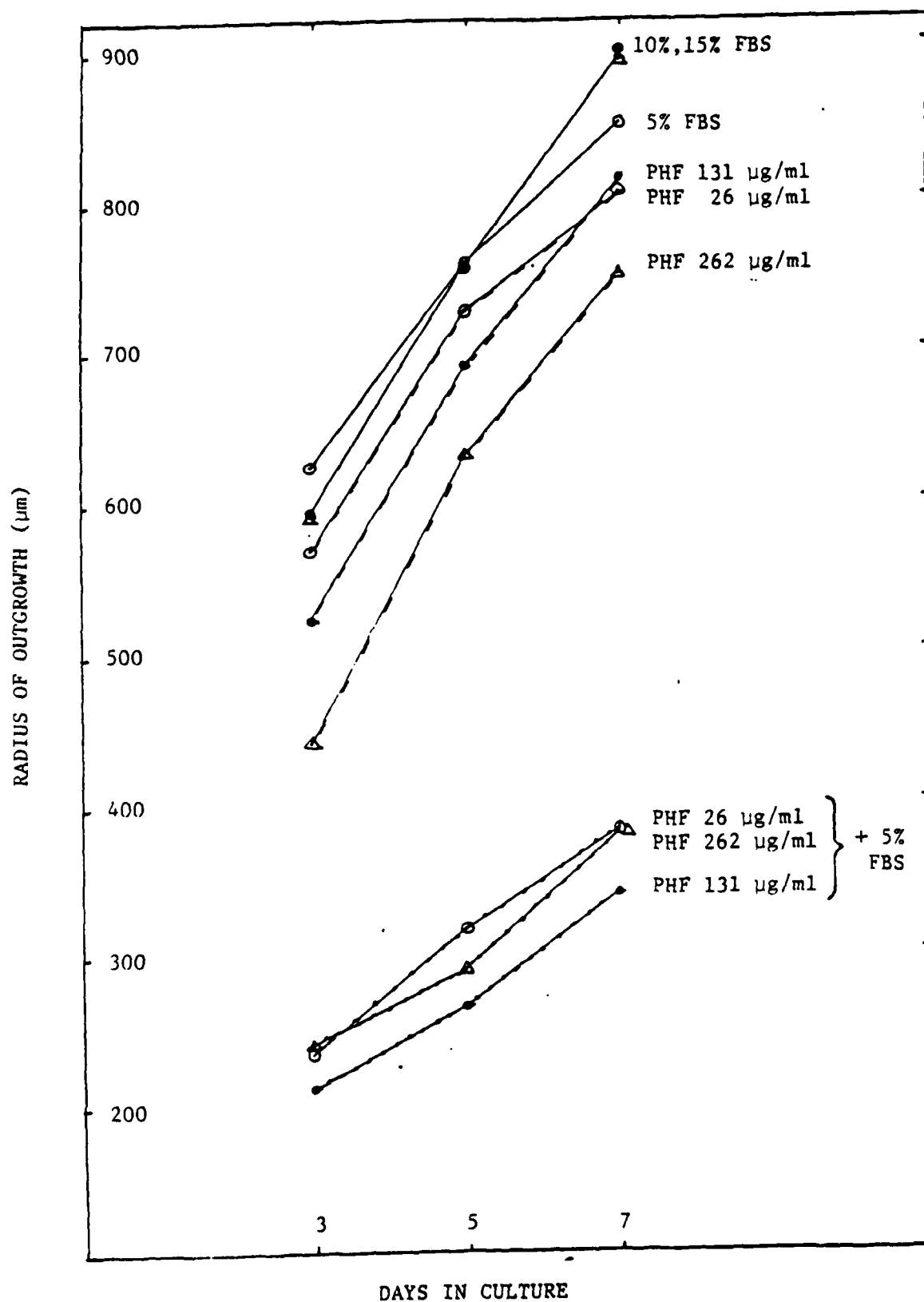
outgrowth of epidermal cells from explants. This factor may be different from the platelet-derived growth factor, since in our studies commercial PDGF alone did not support epidermal outgrowth (Table III). This is the first demonstration to our knowledge of an effect of platelets upon epidermal cells. Since the cultures consist of partial thickness skin including epidermal and dermal tissue, it is not possible using this explant culture model to differentiate between direct effects on the epidermis and indirect effects via the dermis. The potential applications for stimulating epidermal cell growth (migration and mitosis) in injured tissue and the new knowledge of the biochemistry of the skin (regulation of epidermal cell proliferation) make this an exciting area for further investigation. In future experiments, we will use many concentrations of PHF in order to define the linear range of the factor. We will further fractionate the PHF to isolate the factor so that we can determine if it is platelet-derived growth factor or some other known stimulator (such as epibolin), or whether it is a new factor from platelets.

CONCLUSIONS

A. We have preliminary indications of stimulation of epidermal wound healing in vivo in young pig skin with topical application of platelet homogenate (3×10^{10} platelets/ml). In the next three to four months we will conduct a complete animal study to obtain sufficient data for statistical analysis.

B. A component or components from platelet homogenate supports the outgrowth of epidermal cells in skin explant cultures in the absence of fetal bovine serum; this component may be different from platelet-derived growth factor. In addition, there may be another component in platelets that inhibits at high concentrations epidermal cell outgrowth . The physiological roles of these components may be very important in the normal process of wound healing.

RECOMMENDATIONS

Based on the results from the first year, we plan to conduct a complete study of wound healing in the pig model using a daily application of a concentrated platelet homogenate (3×10^{10} platelets/ml). The samples will be analyzed for epidermal resurfacing and collagen biosynthesis. If collagen biosynthesis is not stimulated by the platelet homogenate, we will test for the following possibilities: 1) Collagen biosynthesis is already maximally stimulated by endogenous factor in the wounds. We will test the platelet homogenate fraction in nonwounded skin, by intradermal injections. 2) The factor is not reaching the receptor. Therefore we will try other methods of application such as applying the platelet homogenate under occlusive dressing, injecting sterilized homogenate intradermally around the wound bed or using a viscous vehicle for the platelet factor, such as an ointment-based emollient. 3) The active factor may not be at the optimal concentration. To concentrate the factor the soluble fraction from platelet homogenate will be lyophilized and resuspended in a

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smaller volume of buffer or concentrated with an Amicon filter. This higher concentration will be applied to the wound by the best method as determined in the above experiments.

We are continuing the in vitro explant culture experiments: 1) In additional experimennts we hope to characterize the stimulatory effect that the platelet homogenate fraction (PHF) has upon epidermal cell outgrowth and determine the optimal concentration of the platelet factor. 2) The PHF will be further fractionated to isolate the stimulatory factor (and the possible inhibitor). Heat stability, net charge and molecular weight will be among the methods used to fractionate the homogenate. 3) Tritiated thymidine incorporation will be used to obtain a mitotic index in order to determine the nature of the stimulatory effect, i.e., whether migration or mitosis or both are affected. 4) We will also test the effect of the factor upon pure epidermal cell cultures to distinguish between a direct effect on the epidermis and an indirect effect via dermal interactions.

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APPENDIX 1

A METHOD FOR ESTIMATING COLLAGEN PRODUCTION IN VITRO BY QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF DANSYLATED RADIOACTIVE PROLINE AND HYDROXYPROLINE

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ABSTRACT

A method has been developed for measuring raiolabelled hydroxyproline and proline in non-dialyzable material synthesized by matrix-free cells bv means of thin-layer chromatography. This method was evaluated with labelled standards and with cells from chick embryonic tissues. It was found that this assay has three basic advantages over previously published techniques in that it is less tedious, faster, and not dependent on the use of expensive or highly specialized equipment.

INTRODUCTION

Collagen synthesis is frequently used as an indicator of normal cellular function within connective tissue. Changes in the rate of collagen synthesis or collagen types may be associated with normal embryonic development and with pathological states such as liver fibrosis, scurvy, Osteogenesis imperfecta and Ehlers-Danlos syndrome. A measurement of rate of collagen synthesis should give insight into the cellular function within connective tissues. Methods in the literature for measuring collagen synthesis include assays for hydroxyproline (1-3) and sensitivity to bacterial collagenase (4). Juva and Prockop (1) have described a technique that requires chemical manipulation and careful quantitation. Other approaches involve ion-exchange column chromatography (2) that becomes cumbersome for multiple sample analysis. Tseng, et al. (3) have published a method for the separation and quantitation of radioactive proline, 4-hydroxyproline and 3-hydroxyproline by means of high-voltage paper electrophoresis. All of these methods have been successfully applied; but because of the use of specialized equipment or fractionation procedures that limit the number of analyses and require extensive and tedious bench work, they may not fulfill certain experimental needs. Sensitivity to bacterial collagenase is a useful approach as long as care is taken to use highly purified enzyme free of other proteases.

This report describes a convenient method for determining the rate of collagen synthesis in matrix-free cells from fresh tissues

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which readily lends itself to multiple sample handling, uses inexpensive equipment that is generally available and requires less bench time than the widely used procedure of Juva and Prockop (1). Starting with the total protein hydrolysates, analysis of 36 samples or more may easily be completed within two days. Thin-layer chromatography of dansylated amino acids has been demonstrated to be highly useful in many aspects of protein chemistry. We have utilized this technique to develop an assay procedure for proline and hydroxyproline and have tested it with prepared standards and with biological samples.

METHODS

Materials. Tissues were obtained from 17-day chick embryos, white leghorn strain (Sacks Farms, Evans City, PA). Dansyl chloride, dansylated amino acids and other biochemicals were purchased from Sigma (St. Louis, MO). Chemicals and solvents were reagent grade. L-[^{14}C(U)]Proline, L-4-[^{3}H(G)]hydroxyproline and scintillation counting cocktail Formula 963 were purchased from New England Nuclear (Boston, MA). Earle's Balanced Salt Solution (EBSS)[2], fetal bovine serum (FBS) and trypsin were from GIBCO (Grand Island, NY). Collagenase used to harvest cells from freshly dissected tissue was obtained from Millipore (Freehold, NJ). Silica gel-coated thin-layer chromatography sheets (20 x 20 cm), without fluorescent indicator, were made by Eastman Kodak Co. (Rochester, NY).

Labelling newly synthesized proteins with radioactive proline following dissection. To prepare matrix-free cells, tissues were digested by incubation with collagenase (0.1 mg/ml) and trypsin (0.25%) at 37°C as previously described (5). In general 3 ml of digestion solution/g tissue was used, except for cornea (3 ml/24 corneas) and sternum (3 ml/12 sterna); 1 g wet weight of tissue yielded approximately 10^8 cells. The cells were then resuspended in EBSS plus 2% FBS at 10^7 cells/ml, the cell number was determined using a hemacytometer.

The cell suspension was incubated with [^{14}C]proline (10 µCi/ml) at 37°C in an atmosphere of 5% CO_2 and 95% air with gentle shaking;

1.0-ml aliquots were removed into small glass test tubes at timed intervals for up to 3 h. The cells were separated from medium by centrifugation at 1000 x g for 2 min and were lysed in 1 ml deionized water. The cell lysate and the medium were placed in a boiling water bath for 10 min, cooled on ice, transferred into small dialysis tubing (0.25 cm I.D.) and dialyzed overnight against running tap water.

Analytical procedures. Protein hydrolysates were assayed by the ninhydrin colorimetric method (6). Aqueous aliquots and thin-layer chromatography sections were counted for radioactivity in 20 ml scintillation fluid in a Beckman LS-8000 three-channel scintillation counter with maximal counting efficiency of 94% for the ^{14}C wide window, 79% for the ^{14}C narrow window and 59% for the ^{3}H window. Samples were counted with an error setting of 2%. Dansylation of standard mixtures of amino acids was carried out using established techniques (7), with modifications that are described under Results. Thin-layer chromatography of standard mixtures and samples was performed using a solvent system of benzene/pyridine/acetic acid (16:4:1, v/v) (8), which gives good separation of dansylated proline and hydroxyproline (see Figure 1).

RESULTS

   Optimal conditions for dansylation. A standard solution was
prepared containing [^{14}C]proline (10^5 cpm/ml), [^{3}H]hydroxyproline
(10^5 cpm/ml), 1 mM proline, 1 mM hydroxyproline, and 8 mM amino
acid mixture (hydrolysate of BSA) in 0.01 N HCl. Aliquots of 50 μL
were dansylated with varying ratios of dansyl chloride to free
amino groups from 0.5 to 10.0. The degree of dansylation was
determined as the percentage of radioactivity that migrated from
the origin. The results are shown in Table I. The imino acids
were almost completely labelled (88%) even when dansyl chloride was
limiting. This result is consistent with the very high rate
constant of dansylation reported for proline (9).

   In samples with a low amount of incorporation of labelled
proline, larger aliquots are needed for measuring the radioactive
proline and hydroxyproline. Other components of the dried aliquot
may exhaust the buffering capacity of the sodium bicarbonate and
lower the pH of the dansylation mixture. When the pH is lower than
9.5, the unreactive, protonated form of the amino acid may be
present in too high a ratio (7). Therefore we examined the effect
of pH on the efficiency of dansylation. When the standard
radiolabelled mixture used above was incubated with dansyl chloride
at different pH's, the results indicated incomplete dansylation
under acidic conditions, as shown in Table II. However, it was
found that the ratio of hydroxyproline to total imino acids did not
change significantly between pH 4.5 and 9.5, even when the
efficiency of dansylation was low (at pH 4.5).

41

We conclude that the imino acids proline and hydroxyproline are readily converted to their dansylated derivatives even under conditions that are less than ideal for other amino acids and that even when less than total conversion is achieved, proline and hydroxyproline are dansylated to equal extents; therefore, the values obtained for the ratio of hydroxyproline to total imino acids are accurate.

Assay of collagen synthesis in freshly dissected tissues. Cells were prepared from embryonic chick cornea and sternum and incubated with labelled proline as described in Methods. Cells and media were collected after 10, 20, 30, 60, 90, and 120 min and dialyzed to remove free radioactive proline[3]. Dialysates were transferred to clean tubes and duplicate aliquots, usually 0.3 or 0.4 ml, were pipetted into glass ampules and lyophilized, while a 50 µl aliquot of the dialysate was removed and counted for ^{14}C. For purposes of calculation, the total volume was set equal to 1.0 ml, the volume during dialysis, and this value was used to obtain the total radioactive proline incorporated into macromolecules by 10^7 cells. The duplicate set was held frozen as a safeguard and the other set of samples was hydrolyzed in 6 N HCl, under N_2, at 110°C for 24 h. Hydrochloric acid was removed by drying the samples over NaOH in a vacuum desiccator warmed in a 65°C water bath. Deionized water was added to the residue twice and evaporated to facilitate complete removal of acid. The hydrolysate was redissolved in 1.0 ml water, and two 50 µl aliquots were

removed, one for the ninhydrin amino acid assay and the other for determination of radioactivity.

Aliquots containing an estimated 5000 cpm were transferred into small vials for dansylation and were evaporated under vacuum as above. Twenty μl of 0.2 M NaHCO$_3$ buffer pH 9.5 was added to each vial. The samples were dried by evaporation, redissolved in 50 μl deionized water and the pH tested using 1-μl capillary tubes and pH paper. If the pH was below 7.0, it was adjusted with 0.1 N NaOH or 0.2 M NaHCO$_3$ buffer pH 9.5, until the pH was between 7.0 and 9.5. Then the sample was dried under vacuum and redissolved in 50 μl H$_2$O. The concentration of amino groups was calculated for each aliquot from the ninhydrin assay, and 50 μl or a 5-fold excess of dansyl chloride (5.4 mg/mL acetone) was added. This step is necessary only for initial studies and need not be done for routine analysis when the approximate concentration of amino acids is known, since the ratio of dansyl hydroxyproline to total dansylated imino acids is not altered by incomplete dansylation.

The vials were sealed with teflon-coated septa and screw caps, mixed by vortex and incubated in a 37°C water bath for 4 h. Then the samples were dried in a vacuum desiccator at room temperature. The residues were reconstituted in 50 μl acetone/water (1:1, v/v), 20 μl aliquots were applied to thin-layer chromatography sheets which had been divided into ten 2-cm lanes using a sharp pencil to score the gel coating so as to prevent cross-contamination between lanes. Standards for dansylated proline and hydroxyproline (1-2 μg

in acetone/water, 1:1, v/v) were applied to the first and last lanes of each sheet. The chromatograms were developed in an ascending system for 3-4 h until the solvent front reached the tops of the sheets. Chromatograms were air-dried and examined under uv light to locate the fluorescent dansylated imino acids. Usually the strips were cut to provide three sections: the origin (free imino acids), the dansyl-hydroxyproline band, and the dansyl-proline band. The results in Figure 2 indicate that the rate of collagen secretion following an initial lag time is approximately linear for at least 2 h, while collagen content in the cells appears to reach a plateau after about 1 h of labelling.

Collagen synthesis in various tissues. The assay was used to measure collagen synthesis by matrix-free cells obtained from a variety of tissues that differ in collagen content and production. The matrix-free cells were labelled as described above with [^{14}C]proline for 2 h. The results are shown in Table III. Cornea and tendon have relatively high rates of collagen synthesis, skin and sternum have intermediate rates, while aorta, calvaria, liver and skeletal muscle cells show lower levels of collagen synthesis. That liver cells show measurable collagen synthesis is probably due to the embryonic state of the tissue from which they were obtained.

DISCUSSION

We describe an assay in which freshly isolated cells are cultured for a brief period (< 3 hours) in the presence of radiolabelled proline. The cells and media are then collected, dialyzed and hydrolyzed and radioactive proline and hydroxyproline incorporated into protein are measured as the dansylated imino acids using thin-layer chromatography. For this method to be useful, it must give a consistent ratio of labelled hydroxyproline to total labelled imino acids and thereby a reproducible value for total newly synthesized hydroxyproline in the tissue--a measure of the rate of collagen synthesis. When standard mixtures of $[^{14}C]$proline and $[^3H]$hydroxyproline were used to test the limits of this method, it was found that the results are consistent even when the dansylating agent is limiting, and over a wide pH range. We have also demonstrated that this method provides a reliable estimate of collagen biosynthesis for many different tissues. And since we used the ratio of radiolabelled hydroxyproline to total radiolabelled imino acids, small losses during handling after the initial step do not affect the results. The calculations must be adjusted if applied to basement membrane collagen (type IV) with its higher content of hydroxyproline, but will serve for other known collagens, including type V (11) and the minor cartilage collagens (12), in which hydroxyproline $\approx$ proline ≈ 100 residues/10^3 amino acids ($\pm 10\%$).

The assay may be used for time course measurements of collagen production, as shown in Figure 2, in which case cells and media are analyzed separately. The graphs in Figure 2 are characteristic of secretory proteins and agree with other studies of collagen production (13). This assay may also be used for single determinations of newly made collagen when many different samples are to be analyzed. For that purpose radiolabelled hydroxyproline is measured in cells and media combined and the results are expressed according to Peterkofsky (4) as shown in Table III.

This method should serve as a convenient and sensitive assay with fresh tissues from dissection or biopsy, employing relatively inexpensive and readily available materials and equipment. It may easily be used for many samples and analysis is complete in less than two days. It requires less rigorous manipulation than do other currently available procedures.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1: Thin-Layer Chromatography of Dansylated
[^{14}C]Proline and [^{3}H]Hydroxyproline. Separate standard solutions
of [^{14}C]proline (o——o) and [^{3}H]hydroxyproline (x---x) were

dansylated according to a standardized procedure (7) and separated

by thin-layer chromatography (8). After development the lane was

cut into 2-cm sections and counted with the ^{14}C wide window. The

graph shows complete separation of dansyl-proline and dansyl-

hydroxyproline with no significant amount of substrate remaining at

the origin. The slight amount of ^{3}H-labelled material comigrating

with dansylated-proline is probably due to an impurity in the

[^{3}H]hydroxyproline.

Figure 2: Synthesis of Collagen by Cornea and Sternum Cells.
Matrix-free cells from embryonic chick corneas (A) and sterna (B)

were incubated with [^{14}C]proline (10 µCi/ml) and sampled at various

times for [^{14}C]hydroxyproline in non-dialyzable material. The

values plotted in the graphs are the calculated totals (cpm/ml cell
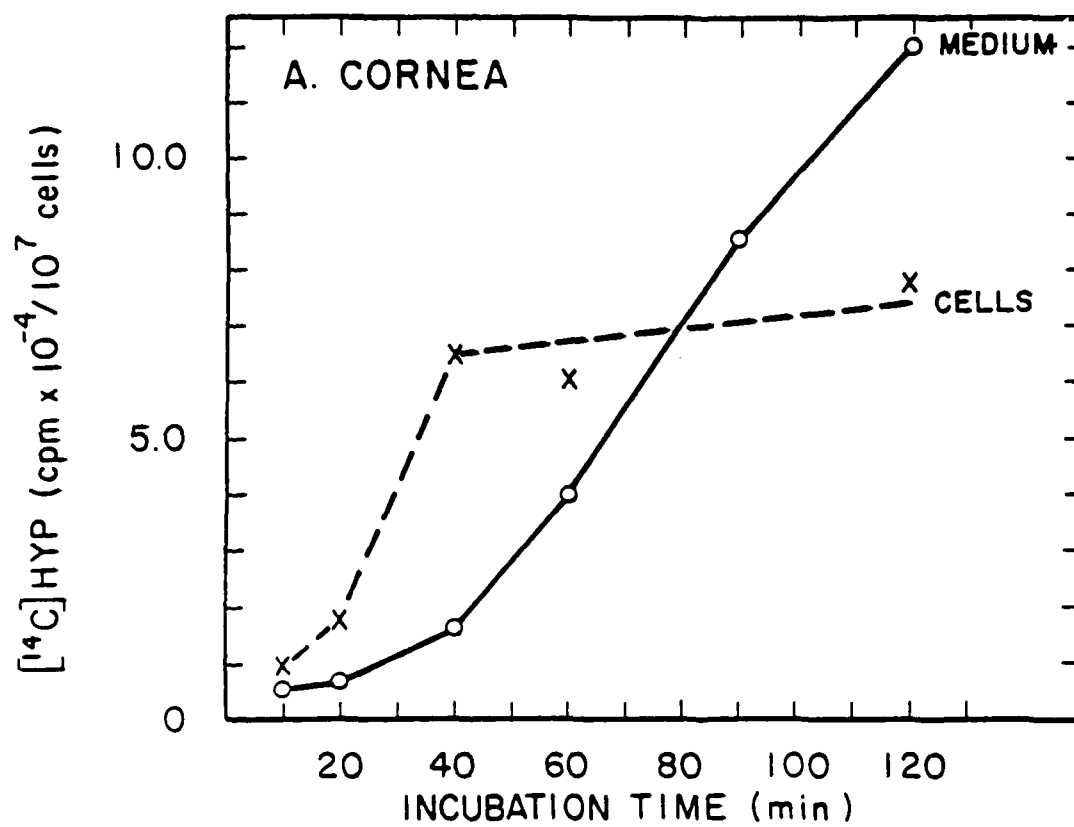
suspension).

P.A. Hebda, W.-Y. Kao

TLC to Quantify Proline
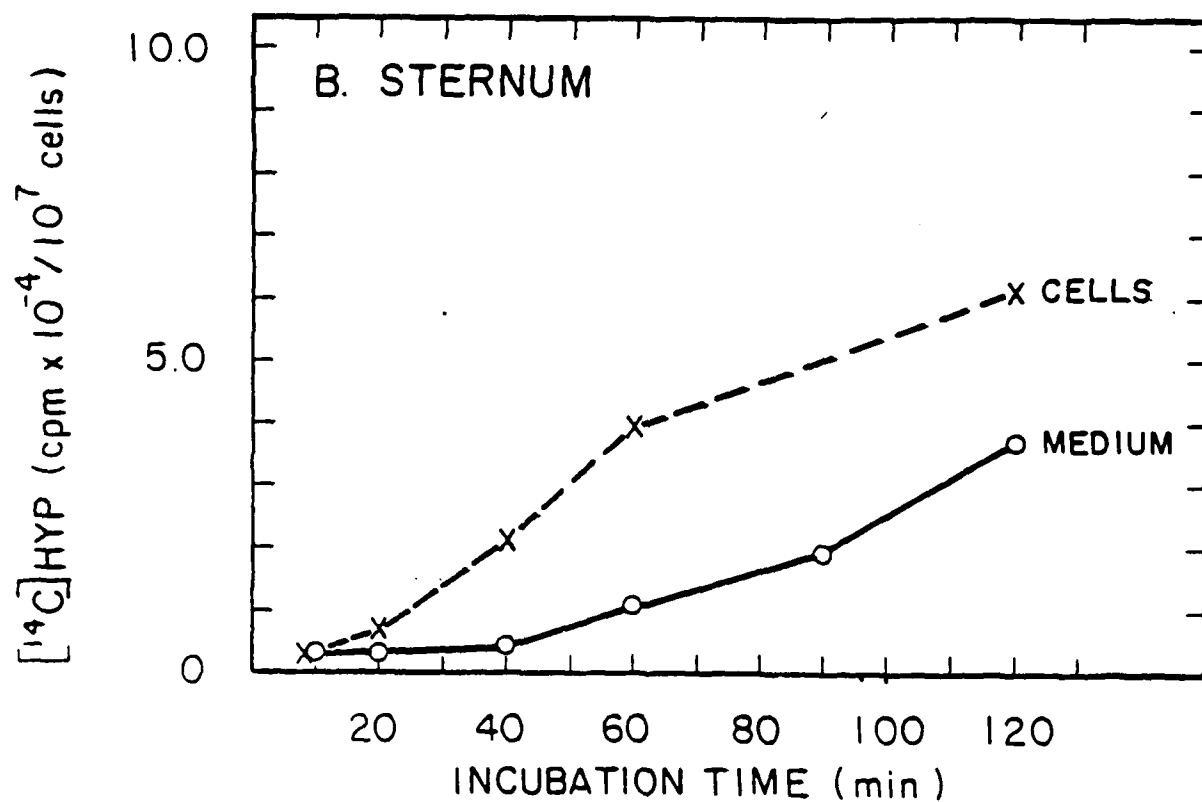and Hydroxyproline

Figure 1   Top ↑

P.A. Hebda, W.W-Y. Kao

TLC to Quantify Proline
and Hydroxyproline

Figure 2A   Top ✝
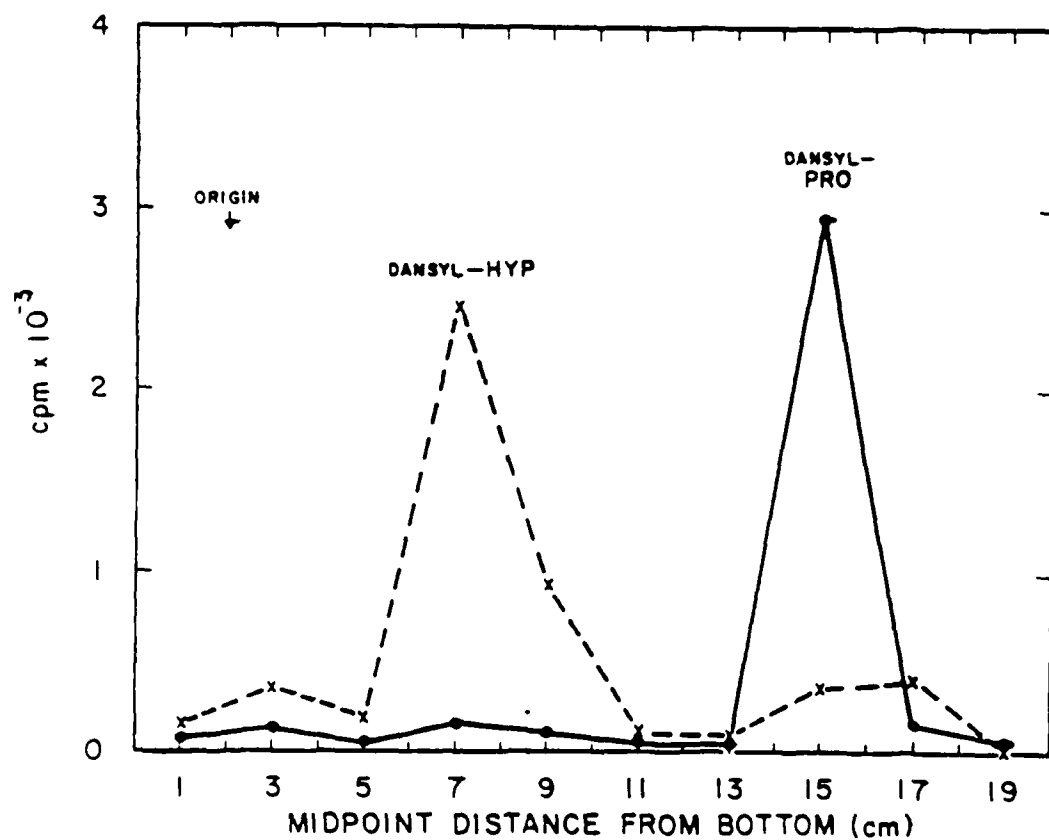
P.A. Hebda, W.W-Y. Kao

TLC to Quantify Proline
and Hydroxyproline

Figure 2B   Top †

B. STERNUM

FOOTNOTES

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2. Abbreviations used in the text are EBSS, Earle's Balanced Salt
   Solution; FBS, fetal bovine serum; BSA, bovine serum alvumin;
   Pro, L-proline; and Hyp, L-4-hydroxyproline.

3. See Peterkofsky and Diegelmann (10) for another method of
   obtaining the total protein fraction from cells.

TABLE I

EFFECT OF VARYING THE AMOUNT OF DANSYLATION REAGENT[o]

Ratio (Dansyl-Cl/Amino Acids)	$\dfrac{Hyp}{Pro + Hyp}$	Efficiency of Dansylation (%)
0.5	0.50	88.4
1.0	0.46	91.6
2.0	0.47	90.4
3.0	0.50	92.0
5.0	0.53	91.0
10.0	0.48	91.9
20.0	0.53	91.4

[o] Samples used for dansylation were taken from a standard solution containing equal amounts (cpm/ml) of [^{14}C]Pro and [^{3}H]Hyp. See Results section of text for details.

TABLE II

EFFECT OF pH ON DANSYLATION EFFICIENCY°

pH	$\dfrac{Hyp}{Pro + Hyp}$	Efficiency of Dansylation (%)
9.5	0.50	89.9
9.0	0.50	90.8
8.0	0.50	91.8
7.0	0.50	89.8
6.0	0.52	84.4
4.5	0.42	02.3

° Samples were prepared as in Table I.

TABLE III

ESTIMATION OF COLLAGEN SYNTHESIS BY CELLS FROM DIFFERENT TISSUES

| Tissue | Protein Synthesis (cpm x 10^{-5}/10^7 cells)[°] | | | | Collagen[§] (%) |
	Total	Collagenous Hyp[‡]	NCP	
aorta	8.45	1.44	5.57	8.7
calvaria	6.44	1.06	4.32	8.3
cornea	9.19	3.05	3.09	26.8
liver	0.95	0.16	0.64	8.2
skeletal muscle	3.53	0.59	2.35	8.5
skin	8.27	2.14	3.99	16.5
sternum	9.32	2.20	4.92	14.2
tendon	10.32	3.52	3.28	28.4

[°] Values are for cells and medium combined

[‡] Noncollagenous protein (total cpm - 2 x Hyp cpm)

[§] $\dfrac{Hyp}{2.7 \times NCP + Hyp}$ x 100%, modified from Peterkofsky (4)

IBRL

PRODUCT PROFILE

PLATELET-DERIVED GROWTH FACTOR
(PDGF)

CATALOG NUMBER 6220LA   Lot No. _____ *11210* _____   Assay Date *12-8-81*
               5 ug

BACKGROUND
: Platelet-derived growth factor was discovered in the early 1970's. It was observed that platelet-poor plasma was deficient in growth promoting activity for various cells in culture; however, addition of an extract from platelets restored this activity to a level comparable to that obtained with clotted, platelet-rich serum.[1-3] PDGF may play a role in wound repair in vivo and stimulates the growth of connective tissue in cells in vitro.[1-6]

: PDGF has recently been purified to homogeneity from clinically outdated human platelets.[7,8] It was found to be a single polypeptide chain with an isoelectric point of 9.8 and a molecular weight of 13,000, probably containing disulfide bonds since it is inactivated by reduction with 2-mercaptoethane

DESCRIPTION
: BRL® PDGF is isolated from clinically outdated human platelets according to the procedure of Antoniades, Scher, and Stiles.[8] The crude preparation is available as a lyophilized solid in 0.1 M Tris HCl, pH 7.4, in 5 ug sizes. The mitogenic activity of the preparation is comparable to that reported by Pledger, Stiles, Antioniades, and Scher.[9] The preparation is active in the nanogram range.

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

SHIPPING/
STORAGE
: BRL® Platelet-Derived Growth Factor is shipped dry and should be stored at -20°C. Under these conditions, the protein is stable for at least six months.

RECONSTITUTION
: Platelet-Derived Growth Factor should be reconstituted in 1 ml of 0.01 M Na_2HPO_4, pH 7.4, 0.15 M NaCl and 1.0% bovine serum albumin* (BRL #5561). If sterility is necessary, reconstituted PDGF can be sterilized by membrane filtration on a non-absorpti membrane. Some loss of protein can be expected.

: *The addition of the bovine serum albumin provides stability to the dilute solution and reduces loss of PDGF by filtration.

: Reconstituted PDGF should be aliquoted and stored in plastic at -20°C for up to two months. Avoid repeated freezing and thawing.

QUALITY CONTROL   The biological potency of BRL® PDGF is given in the figure below. The indicated amount of Human PDGF was added to subconfluent human foreskin fibroblasts made nondividing by culturing under serum-free conditions for the previous 48 hours Sixty hours later, the indicated amount of PDGF was refed to h cells. After 120 hours, the number of cells was counted and th data expressed relative to the mitogenic stimulation of 5% feta bovine serum added to parallel cultures.

$0.1 \, ng/ml =$

$1 \, unit/ml$
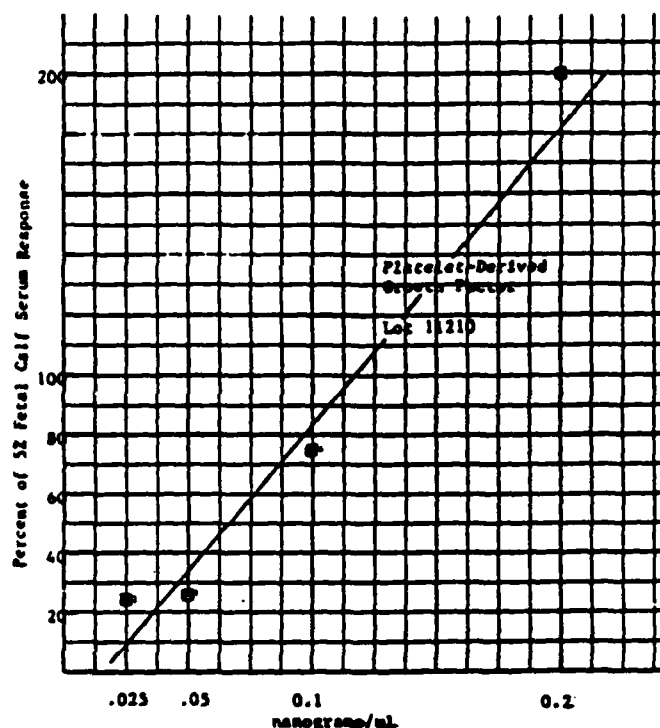
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